# An apyrase from *Mimosa pudica* contains *N*5,*N*10-methenyl tetrahydrofolate and is stimulated by light

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An apyrase (NTP/NDPase) implicated in the response of *Mimosa pudica* to stimuli, such as touch, has been cloned, sequenced and expressed in *Escherichia coli*. While purifying and characterizing this enzyme, it was observed that a chromophore is associated with it, having absorption in the ultraviolet-A/blue region of the spectrum. The absorbance maximum of the chromophore, purified from the enzyme complex by gel filtration and HPLC, is around 350 nm. The chromophore has been identified as *N*5,*N*10-methenyl tetrahydrofolate (MTHF) by comparing the excitation and emission spectra of synthetic MTHF and the isolated cofactor, and by reconstitution of the enzyme complex with synthetic MTHF. Upon excitation with light (350 nm), an increase of apyrase activity was observed in the purified or reconstituted holoenzyme but not in the apoenzyme. The wavelength dependence of the light stimulation matched well with the fluorescence excitation spectra of the cofactor, MTHF. Possible implications of the results for signal transduction in *M. pudica* have been discussed.

*Keywords:* light stimulation; fluorescence; tetrahydrofolate; *Mimosa pudica*.

*Mimosa pudica* is a model sensitive plant where different patterns of responses to the physical, mechanical and chemical stimuli have been demonstrated. Besides the circadian and random spontaneous closing of young leaves of *M. pudica*, when heat, cold, touch, darkness or electric shock is applied, a coherent closing of leaves is noted [1, 2]. Leaf movement organs, called pulvini, are visible as discrete swellings at the base of leaf stalks of legumes. Pulvini, in darkness with their leaves folded, respond to blue light by extending their leaves. Further, pulvini, in light with their leaves extended, respond to darkness by folding the leaves [3].

Light induces various responses in plants. Well-characterized light reception systems in plants are the blue-light receptors. Blue light plays a regulatory role and induces a diverse range of responses in plants. These responses include opening and closing of stomata, chloroplast development, morphogenesis, stimulation of flavonoid biosynthesis and enzymes involved therein [4]. However, the biochemical mechanisms by which plants are able to sense blue light and respond appropriately have not been well understood. Blue-light receptor(s), having characteristics of flavoproteins, have been identified and their association with the signal-transduction elements has already been suggested [5−8]. Recently, putative blue-light photoreceptors from *Arabidopsis thaliana* and *Sinapsis alba* have been shown to have homology to DNA photolyase [9]. Despite the high degree of sequence similarity to photolyases with identical chromophore compositions, neither photoreceptor has photo-reactivating activity.

Our studies indicate that the abundance of an apyrase (NTPase/NDPase) has a positive correlation with the sensitivity of the *M. pudica* leaf to external stimuli such as touch [10, 11]. Further characterization of the apyrase showed that it is a complex of polypeptide and polysaccharide and that this complex formation is mediated through $Ca^{2+}$. The protein moiety devoid of polysaccharide and $Ca^{2+}$ is inactive [12]. The $Ca^{2+}$-modulated activity of NTPase is enhanced in the presence of microtubules and, in addition, tubulin is phosphorylated, implicating the involvement of cytoskeletal proteins in the downstream network of signal transduction (Ghosh, R. and Biswas, S., unpublished results).

While characterizing the NTPase complex further, we noticed that it is associated with a chromophore having absorption in the ultraviolet-A region. This work reports the isolation and identification of the chromophore group of the enzyme complex, as well as the reconstitution of the complex after cloning, sequencing and expression of the NTPase gene in *Escherichia coli.*

## EXPERIMENTAL PROCEDURES

**Materials.** cDNA synthesis kit, cDNA rapid adapter ligation module, cDNA rapid cloning module, mRNA purification kit and [$^{32}$P]dCTP were purchased from Amersham Life Science. Bactoagar, tryptone and yeast extract were purchased from Difco Laboratories. Acrylamide, folinic acid, ampicillin, boric acid, calcium chloride, Tris, BSA, isopropyl-*β*-D-thiogalactoside X-gal and EGTA were purchased from Sigma Chemical Co. 2-Mercaptoethanol was purchased from Aldrich Chemical Co. Triethyl amine was distilled before use. Nitrocellulose paper was from Schleicher & Schüll. All other chemicals and reagents were of analytical grade.

**Methods.** *Construction of cDNA library.* Starting with 5 g leaves, total cellular RNA was prepared from *M. pudica* fresh leaves by a phenol/SDS method [11]. Poly(A)-rich RNA was isolated from total cellular RNA using an oligo(dT)-cellulose

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*Abbreviation.* MTHF, *N*5,*N*10-methenyl tetrahydrofolate.

column supplied in the mRNA purification kit. About 10 μg poly(A)-rich RNA was used for cDNA synthesis. The synthesized blunt end cDNA was ligated with *Eco*RI adaptors using cDNA rapid adaptor ligation module. The adapted cDNA was purified by spin column chromatography. The adapted ends of the cDNA were phosphorylated with ATP (1 mM), and polynucleotide kinase (2 units) prior to ligation with dephosphorylated *Eco*RI λ vector arms.

Dephosphorylated *Eco*RI-cut λ gt11 vector was used for ligation of cDNA. The ligation mixture was incubated at 16 °C for 30 min. The ligation mixture was mixed with the packaging extract and the packaged cDNA was mixed with host strain Y1090, incubated at 37 °C for 15 min and finally plated in presence of 1 mM isopropyl-β-D-thiogalactoside and 1% X-gal on to L-agar/ampicillin (50 μg/ml). The plates were incubated overnight at 37 °C and white plaques were selected as recombinant clones.

*Screening.* The recombinant clones were replica plated on L-agar plates/ampicilin (50 μg/ml), then transferred to nitrocellulose paper for hybridization by the antibody at a dilution of 1:500. Diaminobenzidine was used as a substrate for detection of positive clones. Of the 5000 plaques screened by this method, four turned out to be positive for the antibody. These clones were preserved for further study. Restriction digestion of the clones with *Eco*RI released approximately 2 kb cDNA insert. This insert was then sub-cloned in Bluescript (pBS) for further sequencing.

*Lysogenization. E. coli* Y1089 cells in Luria-Bertani containing 10 mM $MgCl_2$ were infected with recombinant λ gt11 for 15 min at 32 °C and plated on a Luria-Bertani agar plate and incubated at 32 °C. Single-cell colonies were replica plated and incubated overnight at 32 °C and 42 °C. Clones growing at 32 °C only were selected as lysogens [13].

*Expression and detection of recombinant protein. E. coli* cells, bearing recombinant lysogens, were grown overnight at 32 °C with gentle shaking in 10 ml of Luria-Bertani medium containing 0.05 mg/ml ampicilin and isopropyl-β-D-galactoside (10 mM, final concentration). Cells were harvested by centrifugation (5000×*g*, for 5 min), then suspended in 1 ml 10 mM Tris/HCl, pH 7.2, containing 2 mM phenylmethylsulfonyl fluoride, and sonicated (4×30 s, 100 W). Lysates were clarified by centrifugation (12 000×*g* for 15 min). The supernatant was analyzed by SDS/PAGE followed by Western blotting. In Western-blot analysis, rabbit antiserum raised against apyrase of *M. pudica* was used as a primary antibody at a dilution of 1:500. As a secondary antibody, horseradish peroxidase was used at a dilution of 1:5000 and for the color reaction diamino-benzidine was used as the substrate. In the Western blot, in addition to the fusion peptide, another protein band was observed which was 36 kDa (Fig. 1) and corresponds to the protein apyrase (run as a control) from *M. pudica* (data not shown). As a control, wild-type *E. coli* extract was screened with the same antibody. No bands were observed, suggesting the highly specific nature of the antibody.

*Purification of the expressed protein from cloned gene.* The recombinant apyrase from *E. coli* was purified by a protocol similar to that for the plant enzyme [11]. Native gel electrophoresis was carried out with crude cell lysate followed by activity staining with ATP. The band that corresponded to the apyrase (run as a control) from *M. pudica*, was cut out from the gel, electroeluted and NTP-NDPase activity was measured. The recombinant protein was found to be highly active and its characteristics were similar to the protein isolated from the plant *M. pudica*. The purity of the protein was checked by SDS/PAGE, stained with silver stain. The purified protein showed a single band on SDS/PAGE (Fig. 1).

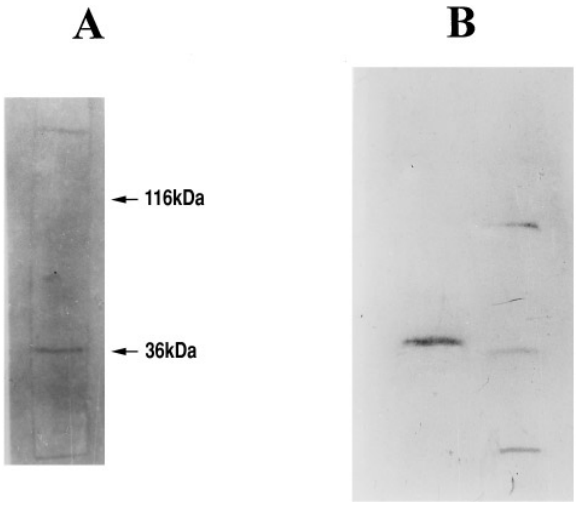


**Fig. 1. Western blot (A) and silver stain (B) of SDS/polyacrylamide gel.** (A) Western blot of SDS/polyacrylamide gel of the total extract (after centrifugation) of *E. coli* cells bearing apyrase cDNA. The positions of the two standards are marked. Approximately 10 μg total protein was loaded. (B) Silver stain of SDS/polyacrylamide gel of the purified recombinant bacterial protein. Approximately 3 μg protein was loaded. The molecular-mass markers from top are 66, 34 and 20 kDa.

*Sequencing of the NTP-NDPase/apyrase gene.* The nucleotide sequence of the cDNA clone was determined by the dideoxy chain termination method of sequencing [14] in an automated DNA sequencer (model 377, semi-adaptive version 3.0) using double-stranded plasmid DNA Bluescript (containing the insert) and the unique forward and reverse primer of Bluescript according to the detailed method supplied.

*N-terminal amino acid sequencing of M. pudica apyrase isoform I.* In order to perform N-terminal amino acid sequencing of *M. pudica* apyrase isoform I, the protein was purified to homogeneity [12]. For sequencing, the protein was dissolved in pure deionized water and, subsequently, the sequencing of the first 20 amino acids was performed by an automated amino acid sequencer (model no 476A, Applied Biosystem Ltd).

*Isolation of the cofactor from the protein.* Apyrase, either from bacterial or plant sources, was treated with 1 mM EGTA for 15 min and passed through a Bio-gel P10 column (20 cm×0.9 cm) equilibrated in 20 mM Tris/HCl, pH 7.0, containing 0.1 M NaCl. The protein was eluted in the first 2−4 fractions, while the cofactor was eluted in the 8−12th fractions (volume 0.75 ml), along with the polysaccharide.

The cofactor was further purified from the polysaccharide component by reverse-phase HPLC (Waters Associates) using a Novapak $C_{18}$ column. The collected material from the gel-filtration column (fractions 8−12) was injected and eluted with 0.1 M triethyl ammonium acetate, pH 7.0, containing 0.6% acetonitrile. The cofactor peak was detected by absorbance at 340 nm and the sugar was detected by refractive index changes (RI monitor).

*Synthesis of* N*5*,N*10-methenyl tetrahydrofolate.* Synthesis of *N*5,*N*10-methenyl tetrahydrofolate (MTHF) was according to Rabinowitz [15]. 50 mg folinic acid was dissolved in 4 ml 1 M 2-mercaptoethanol and the solution adjusted to pH 1.5 by HCl. After incubation at 4 °C for several hours, the precipitate was centrifuged and the supernatant decanted.

*Fluorescence methods.* All fluorescence experiments were done in a Hitachi F3000 spectrofluorometer equipped with a computer for the addition and subtraction of spectra. All fluorescence experiments were performed at 25 °C. The excitation

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1   ATGCTGACCCTGCACCACGTGTCAGACTTGTCAAGAAACGCCTCAAAGAGAAAGAGCTCA
1   M  L  T  L  H  H  V  S  D  L  S  R  N  A  S  K  R  K  S  S
61  ATCGACACAAGAGAGCTAAAATTAGAGGTGACCCACGTATCACACGTTTACACAGTAACA
21  I  D  T  R  E  L  K  L  E  V  T  H  V  S  H  V  Y  T  V  T
121 AGAGATGGAATTTACAATACATTGGGAACATTGGCCGAAGAAGTGAGAAATATCGACTAT
41  R  D  G  I  Y  N  T  L  G  T  L  A  E  E  V  R  N  I  D  Y
181 GACAGAGAGTTCAATAGATGGGAGCACAATAGAAAAAGAACACACCCAAAGAGAAGATTT
61  D  R  E  C  N  R  W  E  H  N  R  K  R  T  H  P  K  R  R  F
241 AGATTTGTGTTTTTCTCATCATGGGACGCAGACACCAACGGCACAATCGACTGCAGAGAG
81  R  F  V  F  F  S  S  W  D  A  D  T  N  G  T  I  D  C  R  E
301 CTCCTCGGAGCAAACGGTGACGGTAAAAGCTCAGCATACAGAGCAGCCGTCGATGGTTAC
101 L  L  G  A  N  G  D  G  K  S  S  A  Y  R  A  A  V  D  G  Y
361 AGAGTGATCTGCGGCGCACACTGGCAGAGACGAGACAGAGACCACAACGGAACAGAGGAC
121 R  V  I  C  G  A  H  W  Q  R  R  D  R  D  H  N  G  T  E  D
421 GAGCACGAGTACGGACAAAGAAGACGGTACCCGGACTACTCATGCACAGCCGGCCACGAC
141 E  H  E  Y  G  Q  R  R  R  Y  P  D  Y  S  C  T  A  G  H  D
481 TCAGATTCAAATAGAACAGAATACAGACACCACAGATTCTCAGGAATCTCAGAGCCCGAC
161 S  D  S  N  R  T  E  Y  R  H  H  R  F  S  G  I  S  E  P  D
541 GGCGACCAAAACTACAGATCATGCCTAGAGAGAGGATCAGAGAGAGACATCCCCTGCTTC
181 G  D  Q  N  Y  R  S  C  L  E  R  G  S  E  R  D  I  P  C  F
601 AGAAAGGCCGAATATCTAAGACATGACGGGGACTCAAACGCCTCATCAGACGACGAGCTC
201 R  K  A  E  Y  L  R  H  D  G  D  S  N  A  S  S  D  D  E  L
661 GGATGTAAGGCGTACGCTTTGATCCTGGCAGAGAACGACTCAAGGAGCTGTAGAGACGGT
221 G  C  K  A  Y  A  L  I  L  A  E  N  D  S  R  S  C  R  D  G
721 GACGGAGAGGTAAATTATGAAGAATTTGTCCAGGTCATGGTCGCAAAATACTCATGGTCG
241 D  G  E  V  N  Y  E  E  F  V  Q  V  M  V  A  K  Y  S  W  S
781 TCAGTACAATCAAACTGGGATGCCGACTGTGAGTCAAACTACTCAGAGGGAGGAAAGGAC
261 S  V  Q  S  N  W  D  A  D  C  E  S  N  Y  S  E  G  G  K  D
841 GGCAGCACAAGAGCACAGTCAAAAGTACAGGATCCCGACAGAAACGACTGCGAAGAACTA
281 G  S  T  R  A  Q  S  K  V  Q  D  P  D  R  N  D  C  E  E  L
901 TTTGCCTCATCACAACACAGCCTTAACCAGCTCTCATGGGCCCTACTGCACGACTCAGAC
301 F  A  S  S  Q  H  S  L  N  Q  L  S  W  A  L  L  H  D  S  D
961 TATAACGCCCTATCAAGCAAGGAGAATGTCCTATGA
321 Y  N  A  L  S  S  K  E  N  V  L  *
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**Fig. 2. The nucleotide and the amino acid sequence of apyrase from *M. pudica*.** Gene bank accession number is AF062398. The bold letters mark the consensus ATP-binding motif and the underlined amino acids indicate the region which has significant similarity with a putative serine/threonine kinase.

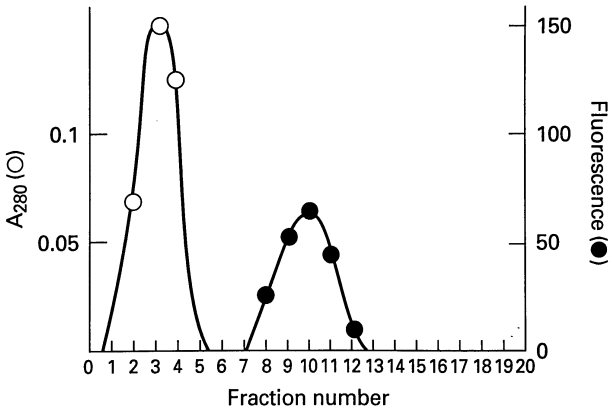


**Fig. 3. Separation of the cofactor from apyrase by gel filtration over Bio-gel P10 by treatment with 1 mM EGTA.** (○) The *A* values of the protein at 280 nm. (●) Represents the cofactor as determined by the fluorescence. Excitation was at 350 nm and the emission maximum at 450 nm was noted. The gel filtration was carried out in 20 mM Tris/HCl, pH 7.0, containing 0.1 M NaCl. The fraction volume was 0.75 ml.

wavelength was at 340 nm. In the case of anisotropy experiments, the excitation wavelength was 350 nm and the emission was fixed at 450 nm. A 5-nm excitation bandpass and 10-nm emission bandpass were used. Appropriate buffer blanks were subtracted from all the fluorescence values. In the polarization studies, the signals were time averaged for 60 s.

*Light stimulation and apyrase assay.* Phosphate assay was carried out as described by Banik and Roy [16]. 1 ml purified apyrase in 20 mM Tris/HCl, pH 7.0, containing 1 mM ATP, at 2 μM (with or without cofactor), was kept in a fluorometer chamber exposed to the desired wavelength of light for 15 min at 37 °C. Then, the whole mixture was added to 1 ml assay buffer (45 μM of $me^7$guanosine, 0.1 unit nucleoside phosphorylase in 20 mM Tris/HCl, pH 7.0) and measured for net loss of fluorescence intensity. The assay temperature was also 37 °C. The excitation wavelength was 300 nm and the emission wavelength was

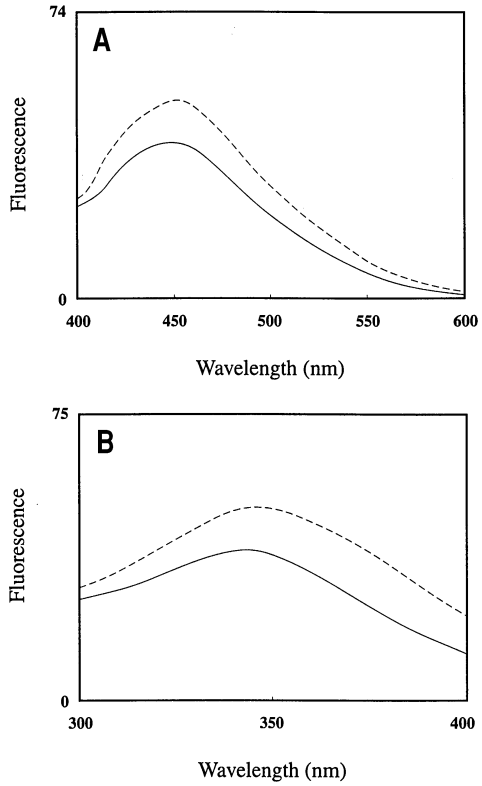


**Fig. 4. (A) Emission and (B) excitation spectra of the purified cofactor and *N*5,*N*10-methenyl tetrahydrofolate.** The excitation wavelength was 350 nm for emission spectra and the emission wavelength was 450 nm for the excitation spectra. Excitation bandpass was 5 nm and the emission band pass was 10 nm. All the spectra were recorded at 25 °C in 10 mM Tris, pH 7.4. (– – –), synthetic MTHF, (——), natural cofactor.

410 nm. The excitation and emission bandpasses were 5 nm and 10 nm, respectively. A phosphate calibration curve was as described in Banik and Roy [16] to convert the fluorescence units to nanomoles phosphate released. One enzyme unit is defined as 1 nanomole phosphate released in 15 min at 37 °C.

## RESULTS

**cDNA sequence of the 36-kDa apyrase (isoform I).** Apyrase (NTPase/NDPase) from *M. pudica* has been purified and partially characterized before [12]. There are two isoforms of the enzyme and all the results reported here have been obtained with isoform I. The first 20 N-terminal amino acids of the apyrase isoform I, as determined with an automated amino acid sequencer, were XLTLHHVSDLSRNASKRKSS. The first cycle was denoted X and contained several peaks including that of the expected amino acid, methionine. The other peaks may have resulted from free amino acid impurities and/or free amino acid contamination. This also suggests that the N-terminus is not blocked or completely processed to remove methionine.

The nucleotide sequence of the 36-kDa apyrase isoform I has been determined (Fig. 2). There are two reasons to believe that the ATG codon (as shown in Fig. 2) is indeed the translation start point. Firstly, the 19 codons after this ATG correspond well with the sequence of the amino acids as determined by amino acid sequencing. Secondly, the open reading frame that ends with a TGA stop codon would encode a polypeptide of 331 amino acids with a calculated molecular mass 36410 Da. This value is in good agreement with the estimated molecular mass of the 36 kDa apyrase (isoform I) isolated from *M. pudica* [12].

**Table 1. Effect of cofactor on stimulation of NTPase activity by blue light.** Apyrase was incubated at 340 nm for 15 min and assayed as described in Experimental Procedures. Reconstituted apyrase means the holoenzyme was reconstituted from apo-enzyme, polysaccharide, $Ca^{2+}$ and the cofactor.

| Nature of the protein | Condition | Activity |
|---|---|---|
| | | units/mg protein |
| Purified apyrase | no exposure | 135 |
| Purified apyrase | 15 min exposure to 340 nm light | 359 |
| Reconstituted apyrase with synthetic MTHF | no exposure | 133 |
| Reconstituted apyrase with synthetic MTHF | 15 min exposure to 340 nm light | 351 |
| Control: apyrase without the cofactor | 15 min exposure to 340 nm light | 122 |
| Control: apyrase without the cofactor | no exposure | 128 |

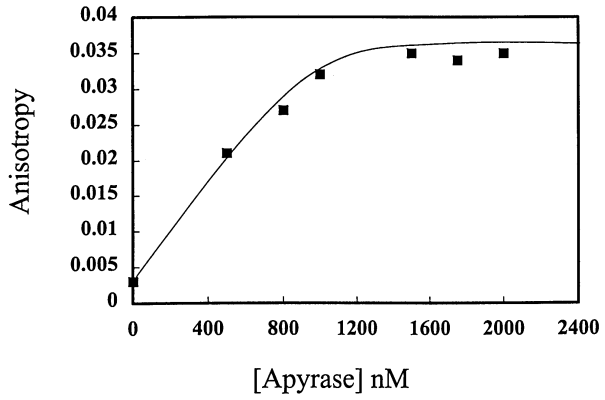


**Fig. 5. Plot of anisotropy versus concentration of protein (depleted of polysaccharide, cofactor and $Ca^{2+}$) to study the binding of synthetic *N*5,*N*10-methenyl tetrahydrofolate with apyrase in presence of calcium.** Increasing amount of apyrase (which is devoid of $Ca^{2+}$, cofactor and the polysaccharide) was added to *N*5,*N*10-methenyl tetrahydrofolate (1 μM) in the presence of 15 μM $Ca^{2+}$. The buffer used was 10 mM Tris, pH 7.4. Excitation was at 350 nm and the emission was at 450 nm.

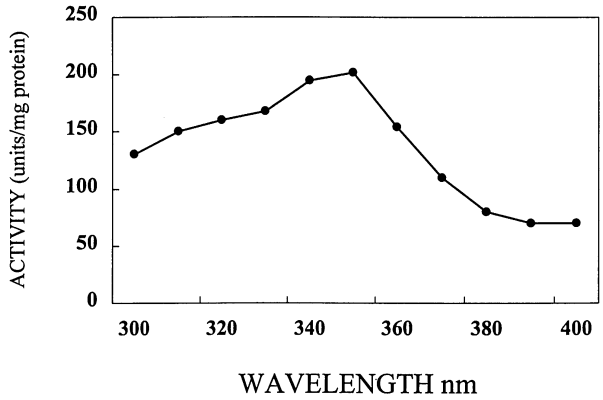


**Fig. 6. Light stimulation of apyrase activity.** The protein (2 μM) in 20 mM Tris/HCl, pH 7.0, containing 1 mM ATP (1 ml) was incubated in the fluorometer chamber in a fluorescence cuvette for 15 min at 37 °C. The excitation wavelength was set at the given value and the shutter was kept closed for 15 min. At the end of that period, the protein was removed and added to the assay mixture (1 ml). The net loss of fluorescence was converted to micromoles phosphate released using a standard curve as described by Banik and Roy [16]. The assay temperature was also 37 °C.

Many ATP-binding proteins [17] contain a consensus ATP-binding domain consisting of GX4GK(S/T)X6(I/V). This conserved sequence has also been found between amino acid residues 103 to 117 of the apyrase. This is consistent with NTP/NDP-binding activity of the apyrase. A BLAST search also revealed significant sequence similarity with a putative Ser/Thr protein kinase in the central portion of the apyrase gene (amino acids 118−119). This is consistent with the observed kinase activity of the protein as mentioned above (Ghosh, R. and Biswas, S., unpublished observation). The central part of the molecule also has extensive similarity with calmodulins from many sources, consistent with known $Ca^{2+}$-binding activity and $Ca^{2+}$-dependent enzymatic activity.

**Characterization of apyrase:** During absorption measurements, we noticed a strong absorption band centered around 340−350 nm, that co-purifies with the protein. This implies the presence of a cofactor, which we attempted to isolate as follows. Realizing the fact that $Ca^{2+}$ probably plays a crucial role in the integrity of the protein, we incubated the purified holoenzyme with a $Ca^{2+}$ chelating agent, EGTA. Upon incubation with EGTA, the protein was chromatographed on a size-exclusion column (Fig. 3). The protein, as characterized by its absorption at 280 nm, is eluted in the void volume. The cofactor, which is fluorescent when excited at 340 nm, is eluted much later, well separated from the protein.

The cofactor at this stage partially co-elutes with the polysaccharide component and has to be purified further by reverse-phase chromatography. The collected and appropriately pooled fractions from size-exclusion chromatography were injected onto a $C_{18}$ column, equilibrated with 0.1 M triethylammonium acetate containing 0.6% acetonitrile and eluted in the same solvent. The elution profile showed one major ultraviolet-absorbing peak and two minor ultraviolet-absorbing peaks. The major ultraviolet absorbing peak was collected, pooled and frozen for further studies. A refractive index detector was used to determine the position of the polysaccharide component. Although the cofactor and the polysaccharide component elute nearby, they can be separated by collecting small volume fractions. In order to determine whether the cofactor is identical to *N*5,*N*10-methenyl tetrahydrofolate, as was found in photolyases [18], we synthesized *N*5,*N*10-methenyl tetrahydrofolate from folinic acid.

As described in the Experimental Procedures, the apyrase cDNA was cloned and expressed in *E. coli* and the protein purified to homogeneity. Absorption spectra showed the presence of the cofactor, and the enzyme was fully active. This suggests that the cofactor is present in *E. coli* and it is not a cofactor that is exclusively found in the plant kingdom. This cofactor was also purified from bacterially derived protein by the method described above. All further spectroscopic characterization was with HPLC-purified cofactors. Thin-layer chromatography of the synthetic and plant-derived cofactor showed a single spot in the chromatogram (data not shown).

The plant-derived and bacteria-derived cofactors and the synthetic MTHF have identical HPLC elution profiles. All the

experiments described below were performed with both the plant and bacterial cofactors and gave identical results. As mentioned before, the cofactor showed significant fluorescence, when excited in the absorption band centered around 350 nm. Fig. 4A shows the emission spectra of the co-factor purified from plant sources along with the emission spectra of the synthetic *N*5,*N*10-methenyl tetrahydrofolate. Both show emission spectra centered around 450 nm with the nature of the emission band being virtually identical. Fig. 4B shows the excitation spectra of the natural co-factor and synthetic *N*5,*N*10-methenyl tetrahydrofolate. The excitation spectra are centered around 350 nm in both cases and show very similar natures.

To conclusively prove that the co-factor is indeed *N*5,*N*10-methenyl tetrahydrofolate, we have attempted to reconstitute the holoenzyme with synthetic *N*5,*N*10-methenyl tetrahydrofolate. The purified natural cofactor can be reconstituted with the apoenzyme in the presence of $Ca^{2+}$. The reconstitution leads to fluorescence enhancement (data not shown). The spectra are very similar. Fig. 5 shows the fluorescence anisotropy as a function of added apo-enzyme. The increase in anisotropy shows saturation behavior, with saturation occurring at about 1 μM protein. Since the cofactor concentration is 1 μM, the nature of the binding suggests very strong interaction between the cofactor and the apoenzyme.

Since the cofactor absorbs in the ultraviolet-A region, we attempted to determine whether the holoenzyme shows any light stimulation of apyrase activity. Table 1 shows the activity of the enzyme when incubated in the presence and in the absence of light. It is clear that ultraviolet-A light clearly stimulates enzyme activity of purified and reconstituted (with synthetic MTHF) apyrase by about 2.5-fold. This is probably a minimum estimate because the whole volume of solution is not illuminated in the fluorometer. In contrast, no light stimulation of enzyme activity is seen in the absence of the cofactor. The wavelength dependence of stimulation of enzyme activity (Fig. 6) matches the excitation spectra of the cofactor well, indicating light absorption by the cofactor is the initial event in light stimulation of apyrase activity.

## DISCUSSION

*M. pudica* is a rare plant that is able to produce rapid mechanical response when stimulated by light, touch or heat. Although understanding at the cellular level has progressed, very little is known about the biochemical origin of such processes. The apyrase from *M. pudica* was isolated many years back but its full characterization and role in the unique physiology has remained unknown. The positive correlation between the abundance of the protein and the sensitivity of *M. pudica* to external stimuli is suggestive of a role for apyrase in the response. Recent work from our laboratory has indicated that this protein is associated with polysaccharide components and that this association requires $Ca^{2+}$ [12]. In this article we have conclusively shown that the protein contains a co-factor which is identified as *N*5,*N*10-methenyl tetrahydrofolate.

The apyrase, capable of light stimulation, is a novel phenomenon. The enzyme is also unique in other ways, for example, (a) tightly associated polysaccharide component and (b) calcium-mediated interaction of *N*5,*N*10-methenyl tetrahydrofolate [12]. The separation of the co-factor from the enzyme leads to its inability to increase the apyrase activity upon excitation with ultraviolet-A light, but no significant loss of basal apyrase activity. This suggests that MTHF is the crucial component of the photo-stimulation phenomenon.

What the physiological role of this light stimulation of apyrase is, remains to be seen and is at present under investigation. Based on the recent identification of *N*5,*N*10-methenyl tetrahydrofolate as a cofactor of DNA photolyases and the realization of the important roles played by this co-factor in the initial photoreception, we speculate that apyrase may play some role in the light response of *M. pudica*.

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## REFERENCES

1. Bose, J. C. (1926) *Nervous mechanisms in plants*, p. 115, Longmans Green and Co. Ltd, London.
2. Bose, D. M. (1960) *Proc. Natl Inst. Sci. India 26B*, 281−304.
3. Satter, R. L., Guggino, S. E., Lonergan, T. A. & Galston, A. W. (1981) *Plant Physiol. 6*, 965−968.
4. Senger, H. (1984) *Blue light effects in biological systems* (Senger, H., ed.) Springer-Verlag, Berlin.
5. Ahmad, M. & Cashmore, A. R. (1993) *Nature 366*, 162−166.
6. Leong, T. Y., Vierstra, R. D. & Briggs, W. R. (1981) *Photochem. Photobiol. 34*, 697−703.
7. Lin, C., Robertson, D. E., Ahmad, M., Raibekas, A. A., Jorns, M. S., Dutton, P. L. & Cashmore, A. R. (1995) *Science 269*, 968−970.
8. Ahmad, M. & Cashmore, A. R. (1997) *Plant J. 11*, 421−427.
9. Malhotra, K., Kim, S. T., Batschauer, A., Dawat, L. & Sancar, A. (1995) *Biochemistry 34*, 6892−6899.
10. Biswas, S. & Bose, D. M. (1972) *Arch. Biochem. Biophys. 148*, 199−207.
11. Mukherjee, J. & Biswas, S. (1980) *Ind. J. Biochem. Biophys. 17*, 452−456.
12. Ghosh, R., Sen, P. C. & Biswas, S. (1998) *Mol. Cell. Biochem. 187*, 47−55.
13. Huynh, T. V., Young, R. A. & Davis, R. W. (1984) *DNA cloning technique, a practical approach* (Glover, D. M., ed.) vol. 1, p. 49, IRL Press, Oxford.
14. Sanger, F., Nicklen, S. & Coulson, A. R. (1977) *Proc. Natl Acad. Sci. USA 74*, 5463−5467.
15. Rabinowitz, J. C. (1963) *Methods Enzymol. 6*, 814−815.
16. Banik, U. & Roy, S. (1990) *Biochem. J. 266*, 611−614.
17. Walker, J. E., Saraste, M., Runswick, M. J. & Gay, N. J. (1982) *EMBO J. 1*, 945−951.
18. Hamm-Alverez, S., Sancar, A. & Rajagopalan, K. V. (1990) *J. Biol. Chem. 265*, 18656−18662.